QUATERNARY AMMONIUM BASES IN THE COELENTERATES[1]

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The stinging organelles or nematocysts of coelenterates appear to serve two functions. By injecting an irritating substance they serve as effective weapons of defense, while accompanying paralyzing action, probably by a different agent or combination of agents, is useful in quieting prey in the process of feeding. In spite of a considerable amount of work over the past fifty years, the chemical nature of the nematocyst toxin is still unknown. Some of the earlier literature has been summarized elsewhere along with an account of some recent work (Welsh, 1956).

Aqueous extracts of nematocyst-bearing tentacles of representatives of each of the three classes of coelenterates, when injected in crabs, produce a preliminary excitation. Spontaneous autotomy of legs may accompany the excitation. After a time the crabs become paralyzed and, if the dose is sufficient, they fail to recover. An injection of tetramethylammonium chloride mimics the paralyzing action of extracts, while an earlier or simultaneous injection of a salt of tetraethylammonium antagonizes the paralyzing actions both of extracts and a tetramethylammonium halide (Welsh, 1956). Since Ackermann, Holtz and Reinwein (1923) had isolated tetramethylammonium hydroxide ("tetramine") from sea anemones, the possibility existed that this substance, or some derivative, was the active paralyzing principle.

Largely through the efforts of Ackermann and co-workers several other quaternary ammonium bases have been isolated from sea anemones and chemically identified. Ackermann, Holtz and Reinwein (1924a) isolated and identified N-methylpyridinium hydroxide from *Actinia equina*, along with a compound tentatively named "actinin." Later, the same authors (1924b) presented evidence that led them to suggest that actinin was probably the alkaloid stachydrine. Ackermann (1927), however, determined actinin to be γ-butyrobetaine, and not stachydrine. Recently Ackermann (1953) found homarine and trigonelline in extracts of the sea anemone, *Anemonia sulcata,* along with an unidentified base which he first named "anemonin," but later (1954) changed to "zoo-anemonin." Evidence for the occurrence of trigonelline in the siphonophore, *Velella spirans,* had been presented earlier by Haurowitz and Waelsch (1926). Zoo-anemonin was identified as the dimethylbetaine of imidazole acetic acid by Ackermann and Janka (1953), but the correctness of the structural formula that they gave will be discussed later.

The present study began as an attempt to determine whether or not tetramine was generally present in coelenterates. Since paper chromatography was used, followed by reagents that help in the visualization of quaternary ammonium bases, it soon became obvious that several such compounds were present. The work was

[1] This investigation was supported in part by research grant B-623 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service.

extended in an attempt to identify these. Unfortunately certain of the papers of Ackermann and others were not known to us until we had spent considerable time in the identification of those bases. We have now identified, with reasonable certainty, tetramethylammonium (I), homarine (III), trigonelline (IV) and γ-butyrobetaine (V) in representatives of all three classes of coelenterates (see Fig. 1). We find what probably corresponds with Ackermann's zoo-anemonin (VI) in a horny coral and in two species of sea anemone. With the method used, we have been unable to identify N-methylpyridinium (II) in any coelenterate, although using the same method we can demonstrate its presence in certain molluscan tissues. Some spots which react as quaternary ammonium bases have not been identified.

Preliminary tests of the toxicity of the identified bases have been made.

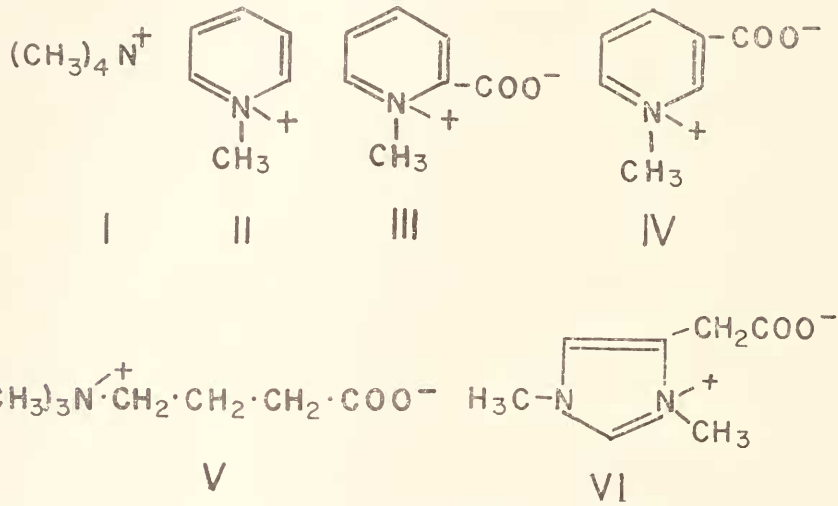

FIGURE 1. Structural formulae of compounds included in this study. I = tetramethylammonium; II = N-methylpyridinium; III = homarine; IV = trigonelline; V = γ-butyrobetaine; VI = zoo-anemonin.

MATERIALS AND METHODS

Extracts were made from the following:

Class Hydrozoa

Hydra littoralis—some supplied by Dr. W. F. Loomis; some mass cultured according to Loomis and Lenhoff (1956); others collected locally.

Physalia physalis L.—the Portuguese man-of-war, fishing filaments only; collected in Bermuda and Bimini, B. W. I.

Class Scyphozoa

Cyanea capillata (L.)—brown or red jellyfish, tentacles only; collected in Puget Sound, Washington.

Class Anthozoa

Plexaura flexuosa—a horny coral, whole animal; collected in Bermuda.
Metridium dianthus (Ellis)—sea anemone, whole animal or tentacles; collected at Nahant and Rockport, Massachusetts.
Condylactis gigantea (Weiland)—pink-tipped sea anemone, tentacles only; collected in Bermuda.

Whole animals were macerated in a Waring Blendor and 4–5 volumes of acetone added. Tentacles were cut off and placed in 4–5 volumes of acetone. The tissues in acetone were stored in a refrigerator. When needed, a given volume (20 or 25 ml.) of the acetone extract was decanted, filtered and the acetone removed under reduced pressure. The remaining material was dried and washed with about 10 ml. of petroleum ether, the bases were then taken up in 1 or 2 ml. of 95% ethanol for chromatography. No attempt was made to secure quantitative yields but the results give a good idea of the relative amounts of different bases that were extracted from a given species.

Extracts and knowns were chromatographed using wide strips of Whatman No. 3 MM filter paper. After trying a variety of acidic and basic solvent systems we found that the most satisfactory separation was obtained with a mixture of 95 parts of 95% ethanol and 5 parts of ammonium hydroxide (28%), as recommended by Bregoff, Roberts and Delwiche (1953). When two-dimensional chromatograms were run, the second solvent system was n-butanol-acetic acid-water (10:3:8–9). The jars were allowed to saturate for at least seven hours and the papers equilibrated 2–3 hours. The ascending method was used. Jars were kept in a chamber in which the temperature was maintained at 25° C. ± 1°. The most satisfactory chromatograms were obtained after runs of 9–10 hours. After drying, the chromatograms were examined under ultraviolet light (short-wave "Mineralight") and any ultraviolet absorbing areas outlined with pencil. Of the several reagents used to visualize the areas occupied by quaternary ammonium compounds, the most generally satisfactory was Dragendorff's solution ($KBiI_4$ reagent) as modified and used by Bregoff, Roberts and Delwiche (1953).

To identify zoo-anemonin, a solvent system consisting of n-butanol-dioxane-water in the proportions of 4:1:5, was also used to permit comparison of the Rf value with that obtained by Ackermann and Janka (1953).

In order to make more certain the identification of homarine and trigonelline, both of which absorb strongly in the ultraviolet, absorption spectra of eluates were compared with those of synthetic compounds using the Cary recording spectrophotometer. Rather large amounts of extracts were placed on paper and run with ethanol-ammonia solvent. Ultraviolet absorbing areas were outlined and a strip was cut from one side for development with $KBiI_4$. The desired areas were cut out and eluted with distilled water. They were appropriately diluted and absorption spectra were obtained. We are greatly indebted to Mr. and Mrs. Paul Brown for their cooperation in this part of the study.

The toxicities of tetramethylammonium bromide, N-methylpyridinium, homarine, trigonelline, γ-butyrobetaine and N,N′-dimethylimidazole acetic acid were determined on the fiddler crab, *Uca pugilator*, from Florida. Each was tested on one or more lots of 5 crabs, by injecting 0.02 or 0.05 ml. of a 1% solution at the base of one of the walking legs.

The known quaternary ammonium standards used in this study were from the following sources: tetramethylammonium bromide, Eastman Organic Chemicals; N-methylpyridinium bromide, kindness of Dr. J. A. Aeschlimann, Hoffmann-La Roche Inc.; homarine, kindness of Dr. E. L. Gasteiger; trigonelline, General Biochemicals Inc.; while γ-butyrobetaine was prepared from γ-carbomethoxypropyltrimethylammonium bromide (generously supplied by Dr. R. W. Fleming, Parke Davis and Co.) after the method suggested by Bregoff, Roberts and Delwiche (1953). A sample of the dimethylbetaine of imidazole acetic acid, as the hydrochloride ($C_7H_{10}O_2N_2 \cdot HCL \cdot H_2O$), was kindly furnished by Dr. D. Ackermann. A second sample was made from imidazole acetic acid (supplied by Dr. H. Bauer, National Institutes of Health) in the laboratory of Dr. R. B. Woodward. The two samples had similar melting points and similar Rf values. Dr. Woodward informs us that the structural formula for anemonin (zoo-anemonin) as given by Ackermann and Janka (1953) is in error and that the correct formula is as given in the series of structural formulae. The more descriptive name for this substance would, therefore, be N,N′-dimethylbetaine of imidazole acetic acid.

Results

Chromatograms

Extracts of tentacles of whole animals of the six selected species, representing each of the three classes of coelenterates, were chromatographed according to the procedure outlined in the section on Methods. Each extract was run many times along with one or more samples of known quaternary ammonium bases. The relative Rf values of these bases are given at the left of Figure 2. All results are for the ethanol-ammonia solvent system. It may be seen that tetramethylammonium bromide (I) gave an Rf value of 0.75; N-methylpyridinium bromide (II) an Rf of 0.64; homarine HCl (III) an Rf of 0.54; trigonelline (IV) an Rf of 0.32; and γ-butyrobetaine bromide (V) an Rf of 0.27. For each species, the compounds found and identified with reasonable certainty, with the exception of N,N′-dimethylbetaine of imidazole acetic acid (VI), are represented by shaded areas.

Tetramine was present in each of the species examined, being the only base found in *Hydra*. The two sea anemones yielded smaller amounts than the other species and in *Metridium* this spot was most distinct when an extract of tentacles, rather than of whole animal, was used. Extracts of the gorgonian, *Plexaura flexuosa*, contained relatively large amounts of tetramine, as suggested by the larger shaded area. It is of interest to note that separate extracts were made of purple and brown varieties of colonies of *Plexaura*. The chromatograms of these extracts were so similar that they are represented by the one set of spots of the four bases that were identified.

In none of the species examined did we find an indication of the presence of N-methylpyridinium. Since the methods employed have enabled us to identify this substance in extracts of certain molluscan tissues, we believe it to be absent, or present in very small amounts, in the coelenterates investigated. Homarine is a compound now known to be widely distributed among marine invertebrates (Gasteiger, Gergen and Haake, 1955). We found it in all five marine species of coelenterates examined. Although present in relatively large amount in our extracts of

Metridium, it was determined with least certainty in the pink-tipped sea anemone, *Condylactis.*

In the ethanol-ammonia solvent, the Rf values of trigonelline and γ-butyrobetaine were so similar that the spots overlapped. In the case of *Plexaura* extracts, where a relatively large amount of trigonelline was present, two-dimensional chromatograms were run. This permitted a clear-cut separation of trigonelline and γ-butyrobetaine. Trigonelline was not found in our extracts of *Physalia,* although it was identified with reasonable certainty in the other four species. Extracts of the pink-tipped sea anemone, *Condylactis,* contained large amounts of γ-butyrobetaine, while extracts of *Physalia* and *Metridium* appeared to lack this substance.

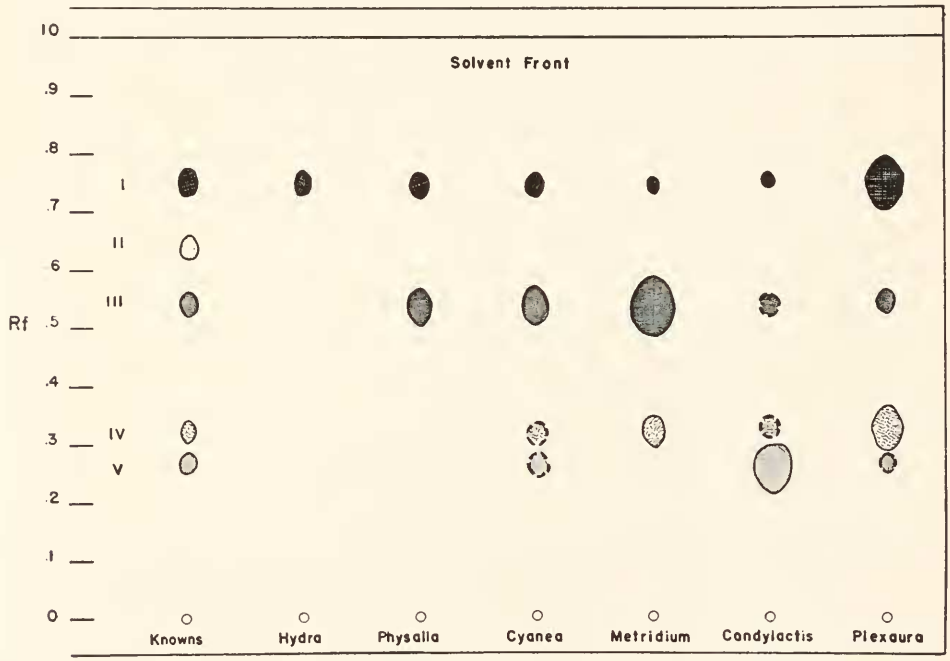

FIGURE 2. Composite of chromatograms giving Rf values for five of the compounds with which this study was concerned. In instances where identification was tentative, the spots are shown with broken boundary lines. I = tetramethylammonium; II = N-methylpyridinium; III = homarine; IV = trigonelline; V = γ-butyrobetaine. Solvent = ethanol-ammonia.

During the period when most of the work reported here was in progress we were not aware of the identification of zoo-anemonin as the dimethylbetaine of imidazole acetic acid. Now having samples of the synthesized material we find what we believe to be zoo-anemonin in *Metridium, Condylactis* and *Plexaura.* Unfortunately, when ethanol-ammonia is used as a solvent system, the Rf value of zoo-anemonin is between 0.2 and 0.3. This is so similar to that for γ-butyrobetaine that some other solvent system must be used for their separation. We have tried n-butanol-dioxane-water (4:1:5) as used by Ackermann and Janka (1953) for zoo-anemonin. With this they obtained an Rf of 0.17. Extracts of *Metridium* and

Plexaura run with this solvent give a relatively large spot appearing between Rf 0.1 and 0.2 and probably representing zoo-anemonin.

Identification by ultraviolet absorption

Spots of N-methylpyridinium, trigonelline and homarine are readily detected as absorbing areas when dried, untreated chromatograms are examined with short-

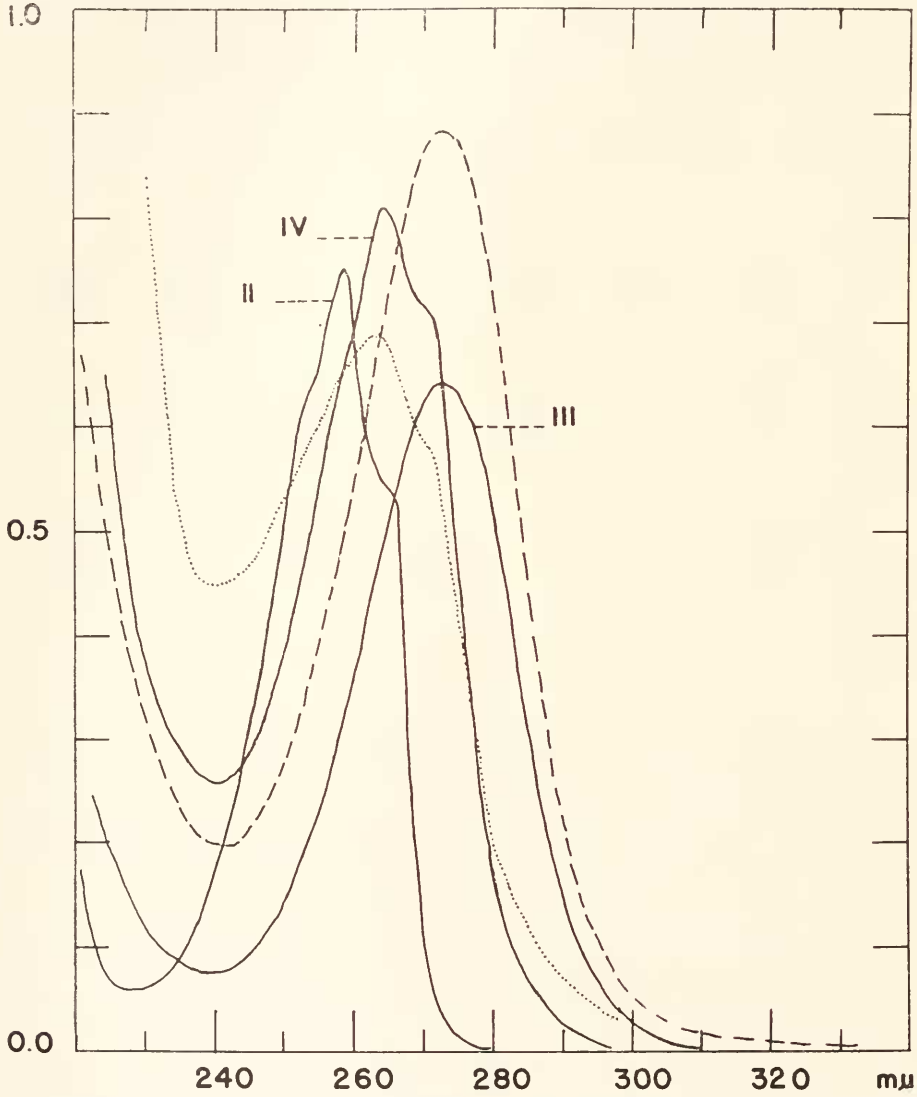

FIGURE 3. Ultraviolet absorption curves for N-methylpyridinium (II), homarine (III), trigonelline (IV), and for eluates of spots from chromatograms of *Metridium* tentacle extracts believed to represent homarine (dashed line) and trigonelline (dotted line). Ordinate = arbitrary units of absorption.

wave ultraviolet light. This was very helpful in the location of regions on the paper where these compounds occurred. Thus, an absorbing area was never found on chromatograms of coelenterate extracts in a region where N-methylpyridinium should have occurred, if it had been present. It should be noted, however, that N-methylpyridinium bromide, when run in ethanol-ammonia, gives two spots, the lower of which is close to homarine. This could give rise to some confusion but we believe, in this instance, that it has not done so.

The ultraviolet-absorbing characteristic of these pyridine derivatives was further used in the identification of homarine and trigonelline. Samples of crystalline N-methylpyridinium, trigonelline and homarine were run in a Cary recording spectrophotometer. Tracings of their absorption curves are combined in Figure 3. Since we were interested mainly in the wave-length at which maximum absorption occurred, their extinction coefficients were not determined. N-methylpyridinium absorbed maximally at 258 mμ, trigonelline at 264 mμ, and homarine at 272–3 mμ.

Extracts of *Metridium* and *Physalia* were streaked on 5-inch-wide strips of paper. After running in ethanol-ammonia the papers were dried and the areas believed to be occupied by trigonelline or homarine were outlined under an ultraviolet light source. These areas were then cut out and eluted with distilled water. After appropriate dilution (determined by trial) the absorption curves for these extracts were also obtained. Figure 3 shows curves for eluates of the areas of *Metridium* chromatograms believed to represent homarine and trigonelline. Maximum absorption of the eluate supposed to contain trigonelline is seen to correspond precisely with that of the authentic samples of trigonelline (IV). The absorption curves for both samples have similar characteristic shoulders.

The absorption curve of a *Metridium* eluate, believed to contain homarine, is identical in shape with that for synthetic homarine. In a similar manner, homarine was identified in extracts of tentacles of *Cyanea* and *Physalia*. The procedure was not used with the other species.

On chromatograms of tentacle extracts of *Metridium* and *Physalia,* run in ethanol-ammonia, an ultraviolet-absorbing area was found with an Rf of about 0.2. Eluates of this area gave absorption curves with a maximum absorption at 248 mμ. The substance responsible for this was not identified.

The action of quaternary ammonium bases on fiddler crabs

Aqueous extracts or homogenates of tentacles of certain coelenterates have been shown to influence the autotomy reflex of crustaceans (Welsh, 1956). Likewise, certain quaternary ammonium bases were found to facilitate or to reduce the tendency to autotomize legs. In order to determine whether or not the bases under investigation in the present study would reproduce the actions of coelenterate extracts, the following experiments were performed. A given volume of *Metridium* tentacles was homogenized with an equal volume of sea water. After centrifuging, 0.05 ml. of the clear supernatant was injected into each of five *Uca pugilator*. In three minutes, six legs had spontaneously autotomized and the crabs were showing signs of severe paralysis, from which none recovered. The original extract was diluted 1:10 with sea water and five crabs injected with 0.05 ml. each. In five minutes, four of the crabs had dropped 25 legs (one crab failed to autotomize any legs). In a few more minutes, they were completely paralyzed and none recovered.

A dilution of 1:100 with sea water produced 12 autotomies in 15 minutes. After 24 hours two crabs were dead and after 48 hours two additional crabs. Thus, an aqueous extract of *Metridium* tentacles contains factors which, in considerable dilution, produce spontaneous autotomy of legs followed by paralysis and death, in this species of crustacean.

An abundance of *Hydra littoralis,* from mass cultures, made it convenient to test the action of a hydra extract on a crustacean. Approximately 2000 hydra, unfed for one week, were blotted and weighed. The wet weight was 410 mg. They were homogenized and one ml. of distilled water added. After centrifuging, 0.02 ml. of the clear supernatant was injected into *Uca pugilator.* The following is a typical record:

1:30 PM	Injected 0.02 ml. at base of second left walking leg of a specimen of *Uca* weighing 3.16 gm.
1:31 PM	First and second left legs "paralyzed"
1:33 PM	Crab cannot right itself when turned on back
1:34 PM	Only slight limb movements
1:40 PM	All spontaneous movements have ceased and no response to stimulation
2:45 PM	No indication of recovery; appears dead, but on removing carapace the heart is found beating.

This extract when diluted 1:10 with sea water was almost as effective in causing paralysis of *Uca* as the undiluted extract. However, when heated at 100° C. for five minutes an injection of 0.02 ml. was entirely without effect on *Uca.*

Next, a series of tests was made to determine whether or not any one of the six bases used in this study, and available in crystalline form, would mimic in any respect the extract of *Metridium* tentacles. Each base was made up as a one per cent solution in sea water and 0.05 ml. injected into each of five *Uca pugilator.* Of the six bases only tetramethylammonium bromide appeared to have significant action. This substance produced a type of paralysis from which only three of five crabs recovered.

It seemed possible that a mixture of the bases in question might have an action that individual members lacked. Therefore, they were combined and injected. The action on the crabs was unspectacular and did not differ from that produced by an equivalent amount of a tetramethylammonium salt. From these injection experiments it would appear that the toxic action of an aqueous extract of *Metridium* tentacles, or whole hydra, on the fiddler crab, *Uca pugilator,* could not be due solely to the presence of the quaternary ammonium bases with which this study was chiefly concerned.

The presence of a tetramethylammonium compound in all species of coelenterates that were examined; its occurrence as the only quaternary base identifiable in *Hydra littoralis* (by the methods used) and its known effects on crustaceans (Welsh, 1956) would all appear to support the earlier suggestion of Ackermann, Holtz and Reinwein (1923) that tetramethylammonium hydroxide (tetramine) might be the paralyzing factor in nematocyst toxin. Two observations made in the present study make this suggestion unlikely. They are (1) that a dose of hydra extract calculated to contain the active material from 0.14 mg. of dry

hydra is fatal to a specimen of *Uca*, while 0.5 mg. of crystalline tetramethylammonium bromide is not, and (2) that heating for 5 minutes at 100° C. destroys or greatly lowers the activity of an aqueous hydra extract. This should have little, if any, effect on a tetramethylammonium salt.

Discussion

Studies made on extracts of whole coelenterates, their tentacles, or their acontia, will not conclusively identify the chemical constituents of nematocyst contents and, therefore, coelenterate or nematocyst toxins, as was recently pointed out by Phillips and Abbott (1957). Such studies may, however, give valuable clues to the nature of the toxic substance, and toxic components of extracts of tissues, rich in nematocysts, may then be sought in extracts of the isolated and cleaned stinging organelles. Methods for isolating undischarged nematocysts have been developed (Phillips, 1956; Phillips and Abbott, 1957) and are being adopted by others (*e.g.* Dodge and Lane, 1958; Lane and Dodge, 1958).

The work reported here was an attempt to learn more about the distribution of tetramine and other quaternary ammonium bases in representative coelenterates. While several bases were found in marine coelenterates, only tetramine was present in the fresh water hydra in sufficient amounts to be identified with the methods employed. This finding, and the observation that tetramine was the only base employed in this study that had significant paralyzing action on *Uca*, provide further evidence that this substance may be a constituent of nematocyst toxin. Almost certainly it is not solely responsible for the paralyzing effects of coelenterate stings. One or more proteins could be additional components. This is suggested by the decreased activity of hydra extracts that have been heated (see above) and by the loss of toxicity by isolated nematocysts that have been treated with ether, alcohol or drying (Phillips and Abbott, 1957). Against the view that proteins may be important in nematocyst toxin is an earlier observation that deproteinization with trichloracetic acid did not significantly alter the toxicity of extracts of acontia of *Adamsia palliata*, when *Carcinus* and *Astacus* were used as test animals (Cantacuzène and Damboviceanu, 1934a). Further observations on the trichloracetic acid extract of *Adamsia* acontia suggest that the crustacean-paralyzing factor is a relatively small and quite stable molecule (Cantacuzène and Damboviceanu, 1934b).

It is not unreasonable to theorize that an association of tetramine with a protein might produce a substance more toxic than tetramine alone. This is based partly on the evidence that two alkylated tetracovalent nitrogens, properly spaced in a molecule, can produce highly active junctional blocking agents such as curare and the many synthetic, curariform, bis-quaternary substances. Protein denaturation by heat, or otherwise, might alter the spacing of the tetramines on the protein or set them free and thereby reduce, but not abolish, the paralyzing action of an extract. In support of such a suggestion is the observation that nematocysts have a high affinity for methylene blue, a basic dye with two methylated nitrogens which, through resonance, may become tetracovalent. This implies that there are molecules within the nematocyst (presumably protein) that bind methylene blue and that might bind other quaternary ammonium bases.

Although certain pyridinium derivatives have a weak curariform action in vertebrates (Craig, 1948) those studied here, as well as the other betaines, were characterized by their lack of paralyzing action on *Uca*. Since homarine, one of the compounds in question, occurs widely in marine invertebrates but not in those from fresh water it has been suggested that it may serve an osmoregulatory function (Gasteiger *et al.,* 1955). This may be the role of some of the other nitrogenous bases of marine invertebrates.

When this study was first begun we tentatively identified one of the bases of *Metridium* and *Physalia* extracts as urocanylcholine (Welsh, 1956). This identification was based partly on the ultraviolet absorption of eluates of chromatograms and their comparison with known urocanylcholine. Although the curves and peaks of absorption correspond rather precisely at a certain pH value, we later learned that the absorption maximum of the eluted material did not change with pH as does that of urocanylcholine (Erspamer and Benati, 1953). Later we learned that the suspected urocanylcholine was actually homarine.

Although we do not yet know what is responsible for the paralysis produced by the nematocysts of coelenterates, the renewed interest in this question should eventually provide an answer.

Summary

This was a study of the identification and distribution of quaternary ammonium bases in representative coelenterates. The purpose was to determine if bases were present with paralyzing actions greater than that of tetramethylammonium (tetramine) which was found to occur in all species examined. Four other bases (homarine, trigonelline, γ-butyrobetaine and the dimethylbetaine of imidazole acetic acid) were found in some species. The bases other than tetramine were found to have no observable paralyzing action on *Uca pugilator,* in the doses employed. However, it is not possible to account for the powerful paralyzing actions of cold, aqueous extracts of *Metridium* tentacles or whole hydra on the basis of their tetramine content. It is suggested that this base, in conjunction with a specific protein, might be responsible for the paralyzing action of nematocysts.

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